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(54) **METHOD OF FORMING OLIGONUCLEOTIDES**

VERFAHREN ZUR DARSTELLUNG VON OLIGONUKLEOTIDEN

PROCEDE DE FORMATION D'OLIGONUCLEOTIDES

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- **NUCLEIC ACIDS RESEARCH**, vol. 21, no. 6, 1993, OXFORD GB, pages 1403-1408, XP002015536 S.M.GRYAZNOV ET AL.: "Template Controlled Coupling and Recombination of Oligonucleotide Blocks Containing Thiophosphoryl Groups."
- **Journal of the American Chemical Society**, Volume 114, Issued 1992, GOODWIN et al., "Template-Directed Synthesis: Use of a Reversible Reaction", pages 9197-9198, see entire document.
- **ALBERTS et al.**, "Molecular Biology of the Cell", published 1983 by Garland Publishing, Inc. (N.Y.), page 187, see entire document.
- **Journal of the American Chemical Society**, Volume 115, issued 1993, GRYAZNOV et al., "Chemical Ligation of Oligonucleotides in the Presence and Absence of a Template", pages 3808-3809.
- **Tetrahedron Letters**, Volume 28, Number 36, issued 1987, THUONG et al., "Synthese et Reactivite D'oligothymidylates Substitues par un Agent Intercalant", pages 4157-4160, see Abstract on page 4157, last sentence.

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Description**TECHNICAL FIELD**

[0001] The present invention relates to a method of forming oligonucleotides and more specifically to methods having use as potential new therapeutic methods for treating viral diseases, cancer, genetic disorders and the like, as well as diagnostic applications of oligonucleotides.

BACKGROUND OF THE INVENTION

[0002] Antisense oligonucleotides have demonstrated potential as new types of therapeutic agents for treating such diseases and disorders as viral diseases, cancer, genetic disorders, as well as other diseases and disorders¹. Extensive research has been carried out and is being continued in industrial and academic laboratories to explore this potential².

[0003] A problem that has been encountered with the approach of utilizing antisense oligonucleotides as therapeutic agents is related to the selectivity of the agents *in vivo*. In view of the low concentrations of intracellular polynucleotide targets and the low concentrations of therapeutic oligonucleotides that can be introduced into cells, it is recognized that there is a need for oligonucleotides with high binding affinities. The binding affinity is related to the length of the oligonucleotides, preferably 20-mers and longer. But, in the case of long oligonucleotides, a mismatch in base pairing is less destabilizing than in the case of a short oligonucleotide. Hence, the desired destabilizing effect is lessened by the use of longer oligonucleotides while the selectivity is increased.

[0004] Experts have noted that "high sequence specificity" and "high affinity" are contradictory demands³. It has further been concluded that on the basis of the extent to which antisense oligonucleotides can cause cleavage of RNAs at imperfectly matched target sites, in systems that were tested it was probably not possible to obtain specific cleavage of an intended target RNA without also causing at least the partial destruction of many non-targeted RNAs⁴. Hence, experts in the field, based on conducted research, have concluded that the conflicting requirements of specificity and affinity are major hurdles to overcome.

[0005] Several methods have been reported for covalently linking oligonucleotide blocks in aqueous media^{5a-1}. All of these methods require an additional chemical agent to yield a stable ligated product. Depending on the approach, the added reagent may be an "activating agent" such as a water soluble carbodiimide or cyanoimidazole^{5a-k} or it may be a reducing agent such as sodium cyanoborohydride^{5l}. In either case, the need for the third reagent precludes chemical ligation *in vivo* since such compounds are toxic, react with water, and could not be introduced into living systems in sufficient amounts to bring about the desired coupling reaction.

[0006] The present invention provides a novel method for covalently linking oligonucleotide blocks present in low concentrations in an aqueous medium without need for an additional condensing or stabilizing reagent. It therefore opens the door for *in situ* chemical ligation in living systems. Since the reactions are greatly accelerated in the presence of a complementary oligonucleotide sequence, one should in principle be able to form long oligonucleotide strands selectively *in vivo* when a target polynucleotide (e.g. m-RNA or DNA from a virus or cancer cell) containing consecutive nucleotide sequences complementary to the individual oligonucleotide strands is present. Long oligonucleotide strands, which bind with high affinity, would therefore be generated *in situ* from shorter strands that bind with lower affinity, when the target polynucleotide is present. This invention could therefore solve the problem of the conflict of achieving high affinity as well as high specificity, in therapeutic and also in diagnostic applications.

SUMMARY OF THE INVENTION

[0007] In accordance with the present invention there is provided a method of forming an oligonucleotide by irreversibly covalently linking at least two oligomers which themselves are reversibly bound by hydrogen bonding at adjacent positions on a target polynucleotide containing a nucleoside base sequence complementary to the sequences of the pair of oligomers, wherein one of the oligonucleotides includes a nucleotide having a first reactive group comprising a bromoacetyl amino adjacent to a nucleotide of the other oligomer which includes a second reactive group comprising a phosphorothioate capable of spontaneously forming a covalent thiophosphoryl acetyl amino bond with the first reactive group. The oligonucleotides are covalently joined together through the first and second reactive groups having been brought into proximity to each other upon binding of the oligonucleotides on the polynucleotide.

[0008] The present invention further provides the use of at least first and second oligonucleotides for preparing a medicament or an oligonucleotide probe, for therapy or diagnosis, characterised in that;

said first oligonucleotide comprises a plurality of nucleotide base units and has a first reactive group comprising a 3' or 5' terminal bromoacetyl amino, said nucleotide base units of said first oligonucleotide being substantially complementary to a first sequence of base units of a target oligonucleotide or polynucleotide;

said second oligonucleotide comprises a plurality of nucleotide base units and has a second reactive group comprising a 5' or 3' terminal phosphorothioate, said nucleotide base units of said second oligonucleotide being substantially complementary to a second sequence of base units of the target oligonucleotide or polynucleotide, wherein said first and second base sequences are at adjacent positions of the target oligonucleotide or polynucleotide; such that said first and second oligonucleotides can be reversibly bound to said adjacent positions of said target oligonucleotide or polynucleotide, whereby said first and second reactive groups are brought into proximity to each other so that a covalent thiophosphorylacetylamino bond can be spontaneously formed between said 3' or 5' bromoacetylamino of said first oligonucleotide and said 5' or 3' phosphorothioate of said second oligonucleotide, in the absence of added reagent.

BRIEF DESCRIPTION OF THE FIGURES

[0009] Other advantages of the present invention will be readily appreciated as the same becomes better understood by reference to the following detailed description when considered in connection with the accompanying drawings wherein:

Figure 1 shows the coupling of two short oligomers in accordance with the present invention utilizing a target template;

Figure 2 shows the facile reaction of an oligonucleotide phosphorothioate with an α -haloacyl oligonucleotide derivative in accordance with the present invention;

Figure 3 shows results of ion exchange high performance liquid chromatography (IE HPLC) of products from experiment 1 wherein: A, after 2 hours in solution at 0°C; B, after 2 days at 0°C; and C, after the final step in which the solution was frozen and stored at -18°C for 5 days, the peaks at approximately 17, 21 and 24 minutes correspond to compounds 1, 2, and 3, respectively.

Figure 4 shows IE HPLC of products from a second experiment (frozen, -18°C throughout) after: wherein A, after 2 hours in solution at 0°C; B, after 2 days at 0°C; and C, after: A, 5 hours; B, 2 days; and C, 5 days, the peaks at approximately 17, 21, and 24 minutes corresponding to compounds 1, 2, and 3, the peak at 27 minutes corresponding to the dimer derivative of compound 2 produced by oxidation by air; and

Figure 5 shows the following: A, IE HPLC of products from the reaction of compounds 1 and 2 in presence of template 4 at 0°C after 2 hours, the major peaks corresponding to coupling product 3 and template 4, noting that compound 1 (peak at 17 minutes) has been almost completely consumed; B, same products after treatment with KI₃ followed by Dithiothreitol (DTT); noting that compound 3 has been replaced by two oligonucleotide cleavage products, eluting at 18 and 22 minutes.

DETAILED DESCRIPTION OF THE INVENTION

[0010] In accordance with the present invention there is provided a method of forming an oligonucleotide generally by the steps of disposing at least two oligonucleotides in aqueous solution wherein one of the oligonucleotides includes a bromoacetylamino group and the other of the nucleotides includes a phosphorothioate group and then covalently binding the oligonucleotides together through the group and the phosphothioate groups spontaneously forming a thiophosphorylacetylamino group therebetween.

[0011] This method exploits the fact that the coupling reaction described herein is very slow in very dilute aqueous solutions but is fast in the presence of a template polynucleotide. That is, the reaction is accelerated in the presence of a target polynucleotide that possesses the sequence section complementary to the probe oligomers. The present invention employs as a therapeutic agent two short oligomers (for example, 8 to 20-mers) which will spontaneously link together covalently after binding at adjacent positions on the target polynucleotide. With this system, one will approach the binding affinity and recognition properties of a longer oligomer probe such as between 16 to 40-mer, but retain the dependency and base pairing characteristics of the shorter probes (8 to 20-mer). In other words, the present invention provides the specificity of shorter polynucleotides while possessing the effect of longer polynucleotides.

[0012] Inherent in the present invention is the need and use of polynucleotides including reactive groups which will spontaneously react to form a covalent bond therebetween when the groups are in spacial proximity to each other. Specifically, the present invention utilizes at least two oligonucleotides wherein one set of oligonucleotides includes the first reactive group and the second set of oligonucleotides include the second reactive group such that upon being brought in proximity to each other, the groups will spontaneously react to form a stable covalent bond. The present invention utilizes a bromoacetylamino group and a thiophosphoryl group, which form a thiophosphorylacetylamino bridge efficiently, selectively, and irreversibly in dilute aqueous media. As demonstrated below, the products are stable in water and hybridize well with complementary polynucleotides.

[0013] At low oligomer concentrations, such as less than 1 μ M, and in absence of a complementary template the

reactions are very slow but can be carried out to high conversion within a few days by freezing the solution. The freezing techniques are described in detail below. Coupling is quite fast (greater than 90% conversion in 20 minutes) when carried out in solution in the presence of a complementary oligonucleotide that serves as a template, as shown below in the Example section.

[0014] Selectivity is also a major concern in diagnostic applications of the present invention and generally in the use of oligonucleotides. The same features of the present invention that make the novel chemistry of the present invention attractive for therapeutic applications also make it attractive for diagnostic uses. For example, the present invention could be utilized in a diagnostic system as follows.

[0015] Referring to Figure 1, A is an oligomer consisting of, for example, a 10-mer bearing a marker (*) in the chain and a bromoacetyl amino group at the 3'-terminus. B is another short oligomer with a thiophosphoryl group at the 5' end. C is a target oligonucleotide sequence with a sequence complementary to A + B. If in dilute solution the coupling of A and B is sufficiently slow in absence of the template, relative to coupling in the presence of the template, only coupling on the template will be significant. This chemical ligation system could therefore be employed in amplification and detection analogously to the enzymatic ligation system (Ligase Chain Reaction, or LCR). It has the potential to be superior since some non-specific coupling introduces a source of error in the enzymatic scheme. The fact that at very low concentrations of oligonucleotides (that is, in the range of interest in diagnostic applications) the rate of the chemical ligation in absence of template becomes extremely slow indicates that the non-template directed coupling could be unimportant in this case.

EXAMPLES

[0016] As shown in Figure 2, the ligation indicated in equation 1 for oligomers 1 and 2 exploits the facile reaction of a phosphorothioate with an α -haloacyl derivative.

[0017] Specifically, compound 1 (Seq. ID 1) in Figure 2 has a 3'-(bromoacetyl amino)-3'-deoxythymidine unit at the 3'-terminus. For preparation of compound 1, 15 μ L of 0.4 M aqueous N-succinimidyl bromoacetate (obtained from Calbiochem) was added to 4.9 A_{260} units of the 3'-aminodeoxyribo-oligonucleotide precursor, ACACCCAATT-NH₂. The method of preparation is described by Gryaznov et al., 1992⁶. The reaction was carried out in 10 μ L of 0.2 M sodium borate buffer at room temperature. After 35 minutes, the mixture was diluted with 0.5 mL of water, desalted by gel filtration on a NAP-5 column (produced by Pharmacia), and purified by RP HPLC high pressure liquid chromatography and again desalted, giving 4 A_{260} units of compound 1. The elution times are as follows: RP HPLC, 17.4 minutes; IE HPLC, 17.4 minutes.

[0018] The IE HPLC carried out above and all similar procedures carried out below was carried out on a Dionex Omni Pak NA100 4x250 mm column at pH 12 (10 mM sodium hydroxide) with a 2% per minute gradient of 1.0 M sodium chloride in 10 M sodium hydroxide. For RP HPLC, a Hypersil ODS column (4.6x200mm) was used with a 1% per minute gradient of acetonitrile in 0.03 M triethylammonium acetate buffer at pH 7.0.

[0019] Compound 2 (Seq. ID 2) was synthesized on a 1 μ mole scale on a Milligen/Bioscience Cyclone DNA Synthesizer using LCAA CPG supported 5'-dimethoxytrityl-N-isobutryldeoxyguanosine. Standard cyanoethyl phosphoramidite chemistry was used. When chain elongation was complete, the terminal 5'-hydroxyl group was phosphitilated (5 minutes) with 150 μ L of a 0.1 M solution of "Phosphate ONTTM" reagent (from Cruachem) in acetonitrile and 150 μ L of 0.5 M tetrazole in acetonitrile. The resulting phosphite was sulfurized by treatment with a 5% solution of sulfur in pyridine/carbon disulfide (1:1, v/v, 45 minutes at room temperature). After cleavage of the DMT group (3% DCA in dichloromethane, 1.5 minutes) the supported polymer was worked up as in the case of compound 1.

[0020] Reaction of a thiophosphoryl-oligonucleotide with a haloacetyl aminoaromatic derivative in DMSO and water has been employed in preparing dye-oligonucleotide conjugates⁷.

[0021] Depending upon the use of the invention and the desired kinetics, coupling of the oligonucleotides can be carried out in either aqueous solution, in a frozen state in ice, or in an aqueous solution in the presence of template, as discussed above and as exemplified below.

[0022] In an initial experiment, 1.0 mL of a solution (pH 7.05, 15 mM phosphate, 85 mM NaCl) containing compounds 1 (0.39 A_{260} units, 4 μ M) and 2 (0.41 A_{260} units, 4 μ M) was prepared and kept at 0°C for 5 days. The solution was warmed to 50°C for 2.5 hours, and finally frozen and stored at -18°C for an additional 5 days. Analysis by IE HPLC of samples after 2 hours and 48 hours showed formation of a slower eluting product, oligomer 3 (Figure 2), in yields of about 25% and 80%, respectively. No significant change was observed after the additional 3 days at 0°C or warming at 50°C. However, the reaction did proceed further in the frozen state, affording a high conversion to compound 3 (Seq. ID 3) within 5 days as shown in Figure 3. The enhanced extent of reaction in the ice matrix may be attributed to the high local concentration of the oligonucleotide reactants within the cavities in the ice. Other reactions have been similarly carried out in an ice matrix⁸.

[0023] In light of this result, an equimolar mixture of compounds 1 and 2 (2 μ M each) in the same buffer was directly frozen and held at -18°C. The HPLC profiles obtained from samples after 5 hours and daily thereafter show progression

to give a high yield of 3 in 5 days, Figure 4 showing representative data.

[0024] Data for coupling compounds 1 and 2 in solution in the presence of a complementary oligonucleotide template (CCATTTTCAGAAATTGGGTGT, compound 4 (Seq. ID 4)) are presented in Figure 5. The system was the same as in the first experiment except template 4 was also present (4 μ M). In this case the reaction proceeded to >90% completion within 20 minutes and was essentially complete within 2 hours.

[0025] The structure assigned to compound 3 is supported by the properties of a model compound (T-NHC(O)CH₂-SP(O)(O⁻)O-T, prepared in solution on a larger scale than used for compound 3), by the mobility of compound 3 on gel electrophoresis (R_m 0.58, compared to R_m 0.89, 0.95, and 0.61 for compounds 1, 2, and 4, respectively), and by the stability of the complex formed with the complementary oligonucleotide, 4. Retention time, RP HPLC 10.5 minutes; FAB⁺ mass spectrum, M+H⁺ 620, M+Na⁺ 642; ³¹P NMR, 6 in D₂O, 18.7 ppm, prior references have disclosed characteristics for the alkylthiophosphate group.⁹

[0026] R_m values are relative to bromophenol blue in a 20% polyacrylamide/5% bis acrylamide gel. The T_m value, 56°C in 0.1 M NaCl, approaches that of the complex formed from the corresponding all-phosphodiester 20-mer and compound 4 (60°C)¹⁰ and differs significantly from values for complexes formed from compounds 1 or 2 with compound 4 (37°C and 31°C). In addition, the internucleotide -NH(CO)CH₂SP(O)(O⁻)- link was found to be cleaved selectively on oxidation with KI₃⁹ (Figure 5). More specifically, the duplex containing compounds 3 and 4 (0.3 A₂₆₀ units each) in 100 μ L of water was treated with 100 μ L of 0.2 M aq. KI₃ for 15 minutes at 50°C. Then 10 μ L of 1 M aq. DTT was added to the solution. After 5 minutes the mixture was desalted on a NAP-5 column and analyzed by IE HPLC.

[0027] The above experimentation provides evidence that the present invention presents novel chemistry which provides a convenient means for selectively and irreversibly coupling oligonucleotides in aqueous solution in the range of 4 μ M oligomer concentration or greater. The products have been shown to be stable in neutral solution and for a few hours even at pH 12 at room temperature. At concentrations below 1 μ M, the rate in the liquid phase become extremely slow. However, the reactions can be carried to near completion in the frozen state. The rate of coupling is markedly accelerated by the presence of a complementary oligonucleotide template. These properties provide a potential in the design of chemical amplification systems and *in situ* ligation in antisense application as well as in building complex structures from oligonucleotide blocks based on known chemistry.

REFERENCES

[0028]

1. (a) Bischofberger, N. and Wagner, R.W. "Antisense Approaches to Antiviral Agents", Virology, 3, 57-66 (1992). (b) Uhlmann, E. and Peyman, A. "Antisense Oligonucleotides: A New Therapeutic Principle" Chemical Reviews, 90, 543-584 (1990).
2. Proceedings, International Conference on Nucleic Acid Medical Applications, Cancun, Mexico, Jan 26-30, 1993; P.O.P. Ts'o and P.S. Miller, Organizers, John Hopkins University, Baltimore, M.D.
3. Proceedings, International Conference on Nucleic Acid Medical Applications, Cancun, Mexico, January, 1993, pg. 60.
4. Woolf, T.M., Melton, D.A., and Jennings, D.G.B. Proc. Natl. Acad. Sci. USA 89, 7305-7309 (1992).
5. (a) Naylor, R.; Gilham, P.T. Biochemistry 1966, 5, 2722-2728. (b) Sokolova, N.I.; Ashirbekova, D.T.; Dolinnaya, N.G.; Shabarova, Z.A. FEBS Letters 1988, 232, 153-155. (c) Shabarova, Z.A. Biochemic 1988, 70, 1323-1334. (d) Chu, B.C.F.; Orgel, L.E. Nucleic Acids Res. 1988, 16, 3671-3691. (e) Kool, E.T. J. Am. Chem. Soc. 1991, 113, 6265-6266. (f) Ashley, G.W.; Kushlan, D.M. Biochemistry 1991, 30, 2927-2933. (g) Luebke, K.J.; Dervan, P.B. J. Am. Chem. Soc. 1991, 113, 7447-7448. (h) Luebke, K.J.; Dervan, P.B. Nucleic Acids Res. 1992, 20, 3005-3009. (i) Prakask, G.; Kool, E.T. J. Am. Chem. Soc. 1992, 114, 3523-3527. (j) Pumal, A.A.; Shabarova, Z.A.; Gumpert, R.I. Nucleic Acids Res. 1992, 20, 3713-3719. (k) Gryaznov, S.M.; Letsinger, R.L., in press, Nucleic Acids Res. (1) Goodwin, J.T.; Lynn, D.G. J. Am. Chem. Soc. 1992, 114, 9197-9198.
6. Gryaznov, S.M., Letsinger, R.L. Nucleic Acids Res., 1992, 20, 3403-3409.
7. (a) Thuong, N.T.; Chassignol, M. Tetrahedron Lett. 1987, 28, 4157-4160. (b) Francois, J.C.; Saison-Behmoaras, T.; Barbier, C.; Chassignol, M.; Thuong, N.T.; Helene, C. Proc. Natl. Acad. Sci. USA 1989, 86, 9702-9706.
8. (a) Beukers, R.; Ylstra, J.; Berends, W. Rec. Trav. Chim. 1958, 77, 729-732. (b) Letsinger, R.L.; Ramsay, O.B.;

McCain, J.H. J. Am. Chem. Soc. 1965, 87, 2945-2953.

9. Mag, M.; Luking, S.; Engels, J.W. Nucleic Acids Res. 1991, 19, 1437-1441.

10. Letsinger, R.L.; Zhang, G.; Sun, D.K.; Ikeuchi, T.; Sarin, P.S. Proc. Natl. Acad. Sci. USA 1989, 86, 6553-6556.

SEQUENCE LISTING

[0029]

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: METHOD OF FORMING OLIGONUCLEOTIDES

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

(A) NAME/KEY: misc_difference
(B) LOCATION: replace(1..11, "")
(D) OTHER INFORMATION: /note= "N is a bromoacetyl amino group"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ACACCCAATT N

11

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

(A) NAME/KEY: misc_difference
(B) LOCATION: replace(1..2, " ")
(D) OTHER INFORMATION: /note= "N is a thiophosphoryl group"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

NCTGAAAATG G

11

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

(A) NAME/KEY: misc_difference
(B) LOCATION: replace (11..12, " ")
(D) OTHER INFORMATION: /note= "NN is a thiophosphorylacetyl amino group"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ACACCCAATT NNCTGAAAAT GG

22

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: both

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc_feature
- (B) LOCATION: 1..20
- (D) OTHER INFORMATION: /note= "Complementary to Seq. 3 without NN"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CCATTTTCAG AATTGGGTGT

20

Claims

1. Use of at least first and second oligonucleotides for preparing a medicament or an oligonucleotide probe, for therapy or diagnosis, **characterised in that:**

said first oligonucleotide comprises a plurality of nucleotide base units and has a first reactive group comprising a 3' or 5' terminal bromoacetyl amino, said nucleotide base units of said first oligonucleotide being substantially complementary to a first sequence of base units of a target oligonucleotide or polynucleotide;

said second oligonucleotide comprises a plurality of nucleotide base units and has a second reactive group comprising a 5' or 3' terminal phosphorothioate, said nucleotide base units of said second oligonucleotide being substantially complementary to a second sequence of base units of the target oligonucleotide or polynucleotide, wherein said first and second base sequences are at adjacent positions of the target oligonucleotide or polynucleotide;

such that said first and second oligonucleotides can be reversibly bound to said adjacent positions of said target oligonucleotide or polynucleotide, whereby said first and second reactive groups are brought into proximity to each other so that a covalent thiophosphorylacetyl amino bond can be spontaneously

formed between said 3' or 5' bromoacetyl amino of said first oligonucleotide and said 5' or 3' phosphorothioate of said second oligonucleotide, in the absence of added reagent.

2. Use according to claim 1, wherein each of said first and second oligonucleotides consists essentially of 8 to 20 nucleotide bases.
3. Use according to claim 1 or 2, wherein said bromoacetyl amino is provided at the 3' terminus of said first oligonucleotide and said phosphorothioate is provided at the 5' terminus of said second oligonucleotide.
4. Use according to claim 3, wherein said first oligonucleotide is ACACCCAATT - NHC(=O)CH₂Br and said second oligonucleotide is SPO₃ - CTGAAAATGG.

5. A product comprising at least first and second oligonucleotides for use as a medicament or an oligonucleotide probe, **characterised in that:**

said first oligonucleotide comprises a plurality of nucleotide base units and has a first reactive group comprising a 3' or 5' terminal bromoacetyl amino, said nucleotide base units of said first oligonucleotide being substantially complementary to a first sequence of base units of a target oligonucleotide or polynucleotide;

said second oligonucleotide comprises a plurality of nucleotide base units and has a second reactive group comprising a 5' or 3' terminal phosphorothioate, said nucleotide base units of said second oligonucleotide being substantially complementary to a second sequence of base units of the target oligonucleotide or polynucleotide, wherein said first and second base sequences are at adjacent positions of the target oligonucleotide or polynucleotide;

such that said first and second oligonucleotides can be reversibly bound to said adjacent positions of said target oligonucleotide or polynucleotide, whereby said first and second reactive groups are brought into proximity to each other so that a covalent thiophosphorylacetyl amino bond can be spontaneously formed between said 3' or 5' bromoacetyl amino of said first oligonucleotide and said 5' or 3' phosphorothioate of said second oligonucleotide, in the absence of added reagent.

6. A product according to claim 5, wherein each of said first and second oligonucleotides consists essentially of 8 to 20 nucleotide bases.

7. A product according to claim 5 or 6, wherein said bromoacetyl amino is provided at the 3' terminus of said first oligonucleotide and said phosphorothioate is provided at the 5' terminus of said second oligonucleotide.

8. A product according to claim 7, wherein said first oligonucleotide is ACACCCAATT - NHC(=O)CH₂Br and said second oligonucleotide is SPO₃ - CTGAAAATGG.

9. A method of forming, *in vitro*, a diagnostic oligonucleotide probe, which method comprises:

(a) reversibly binding at least first and second oligonucleotides to adjacent positions of a target oligonucleotide or polynucleotide, wherein

said first oligonucleotide comprises a plurality of nucleotide base units and has a first reactive group comprising a 3' or 5' terminal bromoacetyl amino, said nucleotide base units of said first oligonucleotide being substantially complementary to a first sequence of base units of said target oligonucleotide or polynucleotide, and

said second oligonucleotide comprises a plurality of nucleotide base units and has a second reactive group comprising 5' or 3' terminal phosphorothioate, said nucleotide base units of said second oligonucleotide being substantially complementary to a second sequence of base units of said target oligonucleotide or polynucleotide, wherein said first and second base sequences are at adjacent positions of said target oligonucleotide or polynucleotide;

(b) covalently joining said first and second oligonucleotides together through the first and second reactive groups having been brought in proximity to each other upon binding of said oligonucleotides to said target oligonucleotide or polynucleotide to spontaneously form a thiophosphorylacetyl amino bond, in the absence of added reagent.

10. A method according to claim 9, wherein steps (a) and (b) occur in aqueous solution.

11. A method according to claim 9 or 10, wherein each of said first and second oligonucleotides consists essentially of 8 to 20 nucleotide bases.

12. A method according to any of claims 9 to 11, wherein said bromoacetyl amino is provided at the 3' terminus of said first oligonucleotide and said phosphorothioate is provided at the 5' terminus of said second oligonucleotide.

13. A method according to claim 12, wherein said first oligonucleotide is ACACCCAATT - NHC(=O)CH₂Br and said second oligonucleotide is SPO₃ - CTGAAAATGG.

Patentansprüche

1. Verwendung von zumindest einem ersten und einem zweiten Oligonucleotid zur Herstellung eines Medikamentes oder einer Oligonucleotidsonde zur Therapie oder Diagnose, **dadurch gekennzeichnet, dass**

- das erste Oligonucleotid eine Vielzahl von Nucleotidbaseneinheiten umfasst und eine erste reaktive Gruppe enthaltend ein 3'- oder 5'-terminales Bromoacetylamin aufweist, und dass die Nucleotidbaseneinheiten des ersten Oligonucleotids im Wesentlichen zu einer ersten Sequenz von Baseneinheiten eines Zieloligonucleotids oder Polyoligonucleotids komplementär sind;

- das zweite Oligonucleotid eine Vielzahl von Nucleotidbaseneinheiten umfasst und eine zweite reaktive Gruppe enthaltend ein 5'- oder 3'-terminales Phosphorothioat aufweist, und dass die Nucleotidbaseneinheiten des zweiten Oligonucleotids im Wesentlichen zu einer zweiten Sequenz von Baseneinheiten des Zieloligonucleotids oder Polynucleotids komplementär sind, wobei die erste und die zweite Basensequenz in benachbarten Positionen des Zieloligonucleotids oder Polynucleotids sind;

- so das erste und das zweite Oligonucleotid reversibel an die benachbarten Positionen des Zieloligonucleotids oder Polynucleotids binden kann, wobei die erste und die zweite reaktive Gruppe in Nähe zueinander gebracht werden, sodass eine kovalente Thiophosphorylacetylaminobindung zwischen dem 3'- oder 5'-Bromoacetylamin des ersten Oligonucleotids und dem 5'- oder 3'-Phosphorothioat des zweiten Oligonucleotids in Abwesenheit von einem zugesetzten Reagenz spontan ausgebildet wird.

2. Verwendung nach Anspruch 1, wobei jedes der beiden des ersten und des zweiten Oligonucleotids im Wesentlichen aus 8 bis 20 Nucleotidbasen besteht.

3. Verwendung nach Anspruch 1 oder 2, wobei das Bromoacetylamin an dem 3'-Terminus des ersten Oligonucleotids und das Phosphorothioat an dem 5'-Terminus des zweiten Oligonucleotids vorgesehen ist.

4. Verwendung nach Anspruch 3, wobei das erste Oligonucleotid ACACCCAATT - NHC(=O)CH₂Br ist und das zweite Oligonucleotid SPO₃ - CTGAAAATGG ist.

5. Produkt umfassend zumindest ein erstes und ein zweites Oligonucleotid zur Verwendung als ein Medikament oder als eine Oligonucleotidsonde, **dadurch gekennzeichnet, dass**

- das erste Oligonucleotid eine Vielzahl von Nucleotidbaseneinheiten umfasst und eine erste reaktive Gruppe enthaltend ein 3'- oder 5'-terminales Bromoacetylamin aufweist, und dass die Nucleotidbaseneinheiten des ersten Oligonucleotids im Wesentlichen zu einer ersten Sequenz von Baseneinheiten eines Zieloligonucleotids oder Polynucleotids komplementär sind;

- das zweite Oligonucleotid eine Vielzahl von Nucleotidbaseneinheiten umfasst und eine zweite reaktive Gruppe enthaltend ein 5'- oder 3'-terminales Phosphorothioat aufweist, und dass die Nucleotidbaseneinheiten des zweiten Oligonucleotids im Wesentlichen zu einer zweiten Sequenz von Baseneinheiten des Zieloligonucleotids oder Polynucleotids komplementär sind, wobei die erste und die zweite Basensequenz in benachbarten Positionen des Zieloligonucleotids oder Polynucleotids sind;

- so das erste und das zweite Oligonucleotid reversibel an die benachbarten Positionen des Zieloligonucleotids oder Polynucleotids binden kann, wobei die erste und die zweite reaktive Gruppe in Nähe zueinander gebracht werden, sodass eine kovalente Thiophosphorylacetylaminobindung zwischen dem 3'- oder 5'-Bromoacetylamin des ersten Oligonucleotids und dem 5'- oder 3'-Phosphorothioat des zweiten Oligonucleotids in Abwesenheit von einem zugesetzten Reagenz spontan ausgebildet wird.

6. Produkt nach Anspruch 5, wobei jedes der beiden des ersten und des zweiten Oligonucleotids im Wesentlichen aus 8 bis 20 Nucleotidbasen besteht.

7. Produkt nach Anspruch 5 oder 6, wobei das Bromoacetylamin an dem 3'-Terminus des ersten Oligonucleotids und das Phosphorothioat an dem 5'-Terminus des zweiten Oligonucleotids vorgesehen ist.

8. Produkt nach Anspruch 7, wobei das erste Oligonucleotid ACACCCAATT - NHC(=O)CH₂Br und das zweite Oli-

gonucleotid SPO_3 - CTGAAAATGG ist.

9. Ein Verfahren zur in vitro Darstellung einer diagnostischen Oligonucleotidsonde, wobei das Verfahren umfasst:

a) ein reversibles Binden zumindest eines ersten und zweiten Oligonucleotids an benachbarte Positionen eines Zieloligonucleotids oder Polynucleotids, wobei

- das erste Oligonucleotid eine Vielzahl von Nucleotidbaseneinheiten umfasst und eine erste reaktive Gruppe enthaltend ein 3'- oder 5'-terminales Bromoacetylamin aufweist, und dass die Nucleotidbaseneinheiten des ersten Oligonucleotids im Wesentlichen zu einer ersten Sequenz von Baseneinheiten des Zieloligonucleotids oder Polynucleotids komplementär sind;

- das zweite Oligonucleotid eine Vielzahl von Nucleotidbaseneinheiten umfasst und eine zweite reaktive Gruppe enthaltend ein 5'- oder 3'-terminales Phosphorothioat aufweist, und dass die Nucleotidbaseneinheiten des zweiten Oligonucleotids im Wesentlichen zu einer zweiten Sequenz von Baseneinheiten des Zieloligonucleotids oder Polynucleotids komplementär sind, wobei die erste und die zweite Basensequenz in benachbarte Positionen des Zieloligonucleotids oder Polyoligonucleotids sind;

b) kovalente Bindungsknüpfung des ersten und des zweiten Oligonucleotids miteinander durch die erste und die zweite reaktive Gruppe, welche durch das Binden der O-ligonucleotide an das Zieloligonucleotid oder Polynucleotid in Nähe zueinander gebracht wurden, um eine kovalente Thiophosphorylacetylaminobindung in Abwesenheit von einem hinzugefügten Reagenz spontan auszubilden.

10. Verfahren nach Anspruch 9, wobei die Schritte a) und b) in wässriger Lösung erfolgen.

11. Verfahren nach Anspruch 9 oder 10, wobei jedes der beiden des ersten und des zweiten Oligonucleotids im Wesentlichen aus 8 bis 20 Nucleotidbasen besteht.

12. Verfahren nach einem der Ansprüche 9 bis 11, wobei das Bromoacetylamin an dem 3'-Terminus des ersten Oligonucleotids und das Phosphorothioat an dem 5'-Terminus des zweiten Oligonucleotids vorgesehen ist.

13. Verfahren nach Anspruch 12, wobei das erste Oligonucleotid ACACCCAATT - $\text{NHC}(=\text{O})\text{CH}_2\text{Br}$ und das zweite Oligonucleotid SPO_3 - CTGAAAATGG ist.

Revendications

1. Utilisation d'au moins un premier oligonucléotide et un second oligonucléotide pour préparer un médicament ou une sonde d'oligonucléotide, pour thérapie ou diagnostic,

caractérisée en ce que

le premier oligonucléotide comprend une pluralité de blocs à base de nucléotide, ainsi qu'un premier groupe réactif comportant un bromoacétylamino terminal 3' ou 5', ces blocs à base de nucléotide du premier oligonucléotide étant essentiellement complémentaires d'une première séquence de blocs de base d'un oligonucléotide cible ou d'un polynucléotide cible ;

le second oligonucléotide comprend une pluralité de blocs à base de nucléotide, ainsi qu'un second groupe réactif comportant un phosphorothioate terminal 5' ou 3', ces blocs à base de nucléotide du second oligonucléotide étant essentiellement complémentaires d'une seconde séquence de blocs de base de l'oligonucléotide cible ou du polynucléotide cible, les première et seconde séquences de base se trouvant dans des positions adjacentes de l'oligonucléotide ou du polynucléotide cible ; de façon que les premier et second oligonucléotides puissent être liés de manière réversible aux positions adjacentes de l'oligonucléotide ou du polynucléotide cible, pour que les premier et second groupes réactifs soient amenés à proximité l'un de l'autre de façon qu'une liaison thiophosphorylacétylamino covalente puisse être formée spontanément entre le bromoacétylamino 3' ou 5' du premier oligonucléotide, et le phosphorothioate 5' ou 3' du second oligonucléotide, en l'absence d'un réactif ajouté.

2. Utilisation selon la revendication 1, dans laquelle chacun des premier et second oligonucléotides est constitué essentiellement de 8 à 20 bases de nucléotide.

3. Utilisation selon la revendication 1 ou 2,
dans laquelle
le bromoacétylamino est prévu à la terminaison 3' du premier oligonucléotide, tandis que le phosphorothioate est
prévu à la terminaison 5' du second oligonucléotide.

4. Utilisation selon la revendication 3,
dans laquelle
le premier oligonucléotide est du ACACCCAATT-NHC (=O)CH₂Br, et le second oligonucléotide est du SPO₃ -
CTGAAAATGG.

5. Produit comprenant au moins un premier oligonucléotide et un second oligonucléotide destinés à être utilisés
comme médicament ou comme sonde d'oligonucléotide,

caractérisé en ce que

le premier oligonucléotide comprend une pluralité de blocs à base de nucléotide, ainsi qu'un premier groupe réactif
comportant un bromoacétylamino à terminaison 3' ou 5', les blocs à base de nucléotide du premier oligonucléotide
étant essentiellement complémentaires d'une première séquence de blocs de base d'un oligonucléotide cible ou
d'un polynucléotide cible ;

le second oligonucléotide comprend une pluralité de blocs à base de nucléotide, ainsi qu'un second groupe réactif
comportant un phosphorothioate à terminaison 5' ou 3', les blocs à base de nucléotide du second oligonucléotide
étant essentiellement complémentaires d'une seconde séquence de blocs de base de l'oligonucléotide ou du po-
lynucéotide visés, les première et seconde séquences de base se trouvant dans des positions adjacentes de
l'oligonucléotide ou du polynucléotide cible ;

de façon que les premier et second oligonucléotides puissent être liés d'une manière réversible aux positions
adjacentes de l'oligonucléotide ou du polynucléotide cible, pour que les premier et second groupes réactifs soient
amenés à proximité l'un de l'autre pour qu'une liaison thiophosphorylacétylamino covalente puisse être formée
spontanément entre le bromoacétylamino 3' ou 5' du premier oligonucléotide, et le phosphorothioate 5' ou 3' du
second oligonucléotide, en l'absence d'un réactif ajouté.

6. Produit selon la revendication 5,
dans lequel
chacun des premier et second oligonucléotides est constitué essentiellement de 8 à 20 bases de nucléotide.

7. Produit selon la revendication 5 ou 6,
dans lequel
le bromoacétylamino est prévu à la terminaison 3' du premier oligonucléotide, et le phosphorothioate est prévu à
la terminaison 5' du second oligonucléotide.

8. Produit selon la revendication 7,
dans lequel
le premier oligonucléotide est du ACACCCAATT-NHC (=O)CH₂Br. tandis que le second oligonucléotide est du
SPO₃ - CTGAAAATGG.

9. Procédé de formation, in vitro d'une sonde d'oligonucléotide de diagnostic,
ce procédé comprenant
les étapes consistant à :

(a) lier de manière réversible au moins un premier oligonucléotide et un second oligonucléotide à des positions
adjacentes d'un oligonucléotide ou d'un polynucléotide cible,

de façon que le premier oligonucléotide comprenne une pluralité de blocs à base de nucléotide, ainsi qu'un
premier groupe réactif comportant un bromoacétylamino à terminaison 3' ou 5', les blocs à base de nucléotide
du premier oligonucléotide étant essentiellement complémentaires d'une première séquence de blocs de base
de l'oligonucléotide ou du polynucléotide cible, et

le second oligonucléotide comprenant une pluralité de blocs à base de nucléotide, ainsi qu'un second groupe
réactif comportant un phosphorothioate à terminaison 5' ou 3', les blocs à base de nucléotide du second
oligonucléotide étant essentiellement complémentaires d'une seconde séquence de blocs de base de l'oligo-
nucléotide ou du polynucléotide cible, les première et seconde séquences de base se trouvant dans des
positions adjacentes de l'oligonucléotide ou du polynucléotide cible ;

(b) lier, par une liaison de covalence, les premier et second oligonucléotides l'un à l'autre par l'intermédiaire

des premier et second groupes réactifs ayant été amenés à proximité l'un de l'autre au moment de la liaison des oligonucléotides avec l'oligonucléotide ou le polynucléotide cible, de manière à former spontanément une liaison thiophosphorylacétylamino, en l'absence d'un réactif ajouté.

- 5 **10.** Procédé selon la revendication 9,
dans lequel
les étapes (a) et (b) se produisent dans une solution aqueuse.
- 10 **11.** Procédé selon la revendication 9 ou 10,
dans lequel
chacun des premier et second oligonucléotides est constitué essentiellement de 8 à 20 bases de nucléotide.
- 15 **12.** Procédé selon l'une quelconque des revendications 9 à 11,
dans lequel
le bromoacétylamino est prévu à la terminaison 3' du premier oligonucléotide, et le phosphorothioate est prévu à la terminaison 5' du second oligonucléotide.
- 20 **13.** Procédé selon la revendication 12,
dans lequel
le premier oligonucléotide est du ACACCCAATT - NHC(=O)CH₂Br tandis que le second oligonucléotide est du SPO₃ - CTGAAAATGG.

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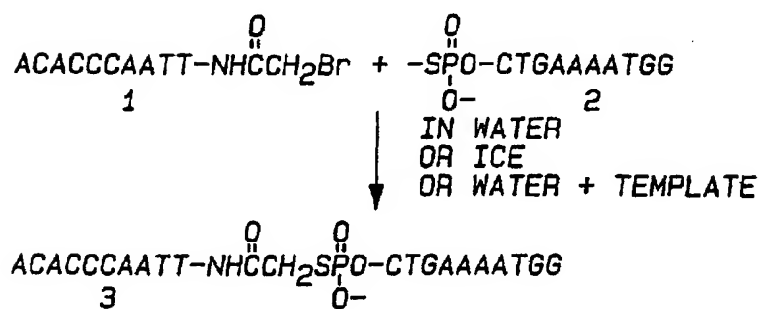
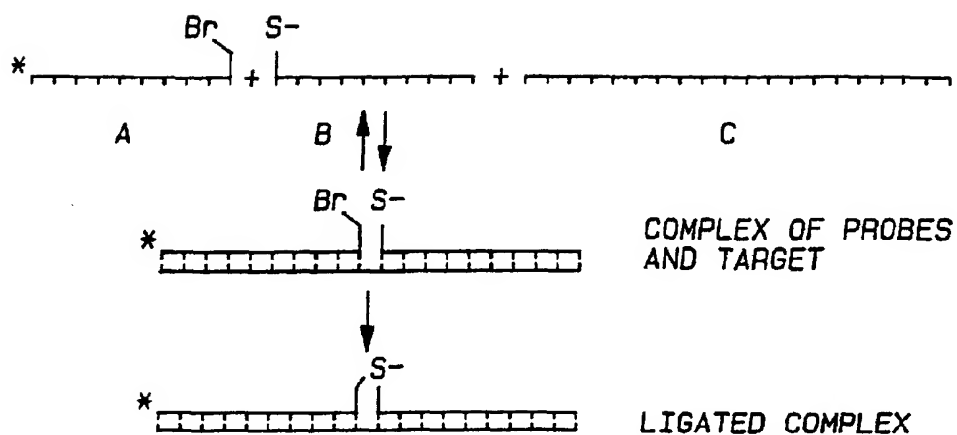
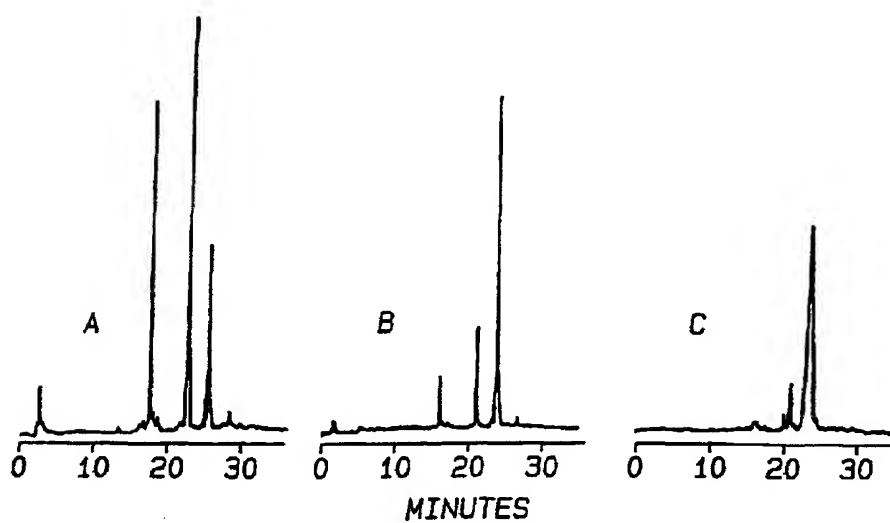


Fig-2



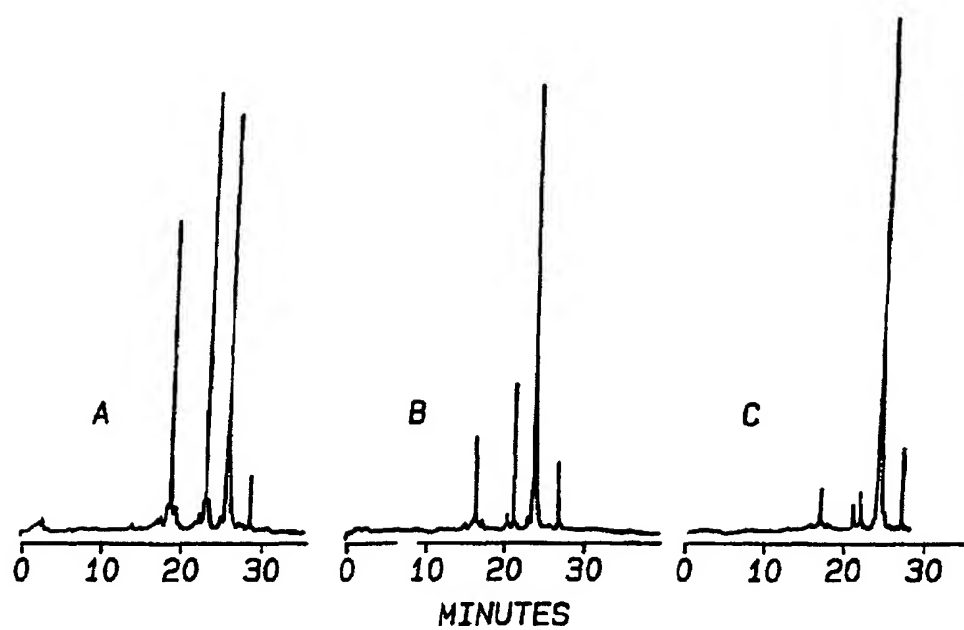


Fig-4

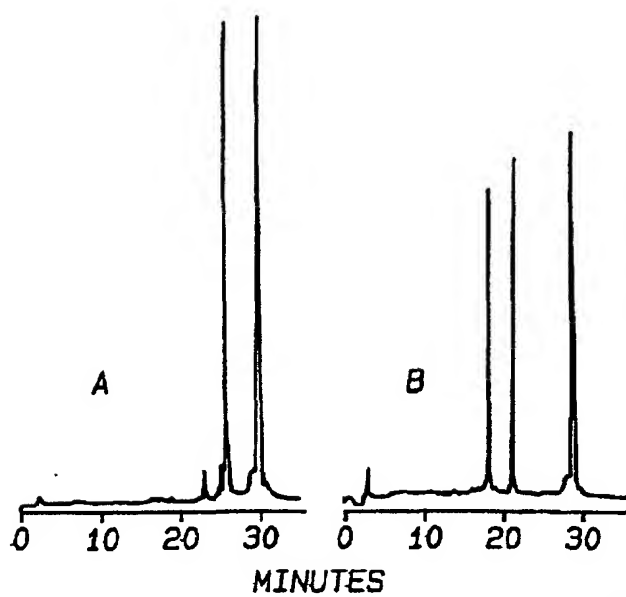


Fig-5